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Regulation of leaf senescence in Arabidopsis

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Chapter 2
***Arabidopsis onset of leaf death* mutants identify a regulatory pathway controlling leaf senescence**

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Abstract
The onset of leaf senescence is controlled by leaf age and ethylene can promote leaf senescence within a specific age window. We exploited the interaction between leaf age and ethylene and isolated mutants with altered leaf senescence that are named as *onset of leaf death* (*old*) mutants. Early leaf senescence mutants representing three genetic loci were selected and their senescence syndromes characterised using phenotypical, physiological and molecular markers. *old1* is represented by three recessive alleles and displayed earlier senescence both in air and upon ethylene exposure. The etiolated *old1* seedlings exhibited a hypersensitive triple response. *old2* is a dominant trait and the mutant plants were indistinguishable from the wild-type when grown in air but showed an earlier senescence syndrome upon ethylene treatment. *old3* is a semi-dominant trait and its earlier onset of senescence is independent of ethylene treatment. Analyses of the chlorophyll degradation, ion leakage and *SAG* expression showed that leaf senescence was advanced in ethylene-treated *old2* plants and in both air-grown and ethylene-treated *old1* and *old3* plants. Epistatic analysis indicated that *OLD1* might act downstream of *OLD2* and upstream of *OLD3* and mediate the interaction between leaf age and ethylene. A genetic model was proposed that links the three *OLD* genes and ethylene into a regulatory pathway controlling the onset of leaf senescence.

Key words: *Arabidopsis,* ageing, ethylene, leaf senescence, *old* mutants, senescence-associated genes

Introduction
Leaf senescence is the final phase of leaf development and in nature it is evident by the golden autumn colours. During senescence a salvage process is activated by which nutrients are mobilised from the senescing leaf to other parts of the plant, such as young leaves and/or developing seeds (Nooden, 1988). For this, leaf cells undergo orchestrated changes in morphology, structure and metabolism, which is referred to as the senescence syndrome (for a review, see Bleecker and Patterson, 1997). Leaf senescence is visibly marked by the colour change from green to yellow and red, which results both from the preferential degradation of chlorophylls compared to carotenoids, and from the concomitant synthesis of anthocyanins and phenolics (reviewed by Matile et al., 1999). In *Arabidopsis thaliana*, the visible yellowing and the chlorophyll loss are widely used to stage the progression of senescence, which reproducibly correlate with other biochemical changes that occur during leaf senescence (Hensel et al., 1993; Lohman et al., 1994).

The execution and completion of senescence requires *de novo* transcriptional and translational activities (for a review see Nooden, 1988). Genes whose transcriptional levels are specifically up-regulated in senescing leaves have been cloned and are collectively termed as senescence-associated genes (*SAG*s) (reviewed by Buchanan-Wallaston, 1997; Nam, 1997). In *Arabidopsis* there are dozens of *SAG*s and their expression patterns have been studied in age-regulated senescence and under various induction conditions. These *SAG*s serve as informative molecular markers for monitoring the progression of senescence. However, genes that define the onset of senescence and those that regulate the expression of these *SAG*s, have not been well characterised yet.

Various factors influence the onset of leaf senescence. Leaf senescence is an intrinsic age-dependent process, and its onset appears to be regulated by the age of individual leaves (Hensel et al., 1993). Senescence is also prematurely induced by a range of external factors such as darkness, detachment, drought and pathogen attack (Park et al., 1998; Weaver et al., 1998; Pontier et al., 1999; Quirino et al., 1999). Under these induction conditions distinct sets of *SAG*s are induced, suggesting the existence of a complex regulatory network. In addition, plant hormones have been implicated in regulating leaf senescence (Gan and Amasino, 1995; Morris et al., 2000; He et al., 2002).

The role of the volatile phytohormone ethylene in leaf senescence is revealed by studies on ethylene-treated plants and ethylene mutants as well as on transgenic plants. On the one hand, ethylene promotes leaf senescence. In wild-type *Arabidopsis* plants, ethylene treatment can advance the visible yellowing and *SAG* induction in leaves that are primed to senesce (Grbic and Bleecker, 1995; Weaver et al., 1998). *Arabidopsis* ethylene-insensitive mutants, exemplified by *etr1*, *ein2* and *ein3*, display delayed leaf senescence (Bleecker et al., 1988; Grbic and Bleecker, 1995; Chao et al., 1997; Oh et al., 1997; Hua and Meyerowitz, 1998). On the other hand, ethylene is neither necessary nor sufficient for the occurrence of senescence. Senescence eventually occurs in the ethylene insensitive mutants. Ethylene constitutive response and overproduction mutants such as *ctr* and *eto* do not show premature leaf senescence as could be expected (Guzmàn and Ecker, 1990; Kieber et al., 1993). In tomato plants carrying *Never ripe* mutation or with the antisense suppression of the ACC oxidase, the progress of leaf senescence is not retarded once senescence has started (Lanahan et al., 1994; John et al., 1995). Together, these studies suggest that ethylene does not directly regulate the onset of leaf senescence. It acts to modulate the timing of leaf senescence.

Our research aims to identify genes that control the onset of leaf senescence. Such genes can be approached by isolating mutants that show altered senescence phenotypes. Oh et al. (1997) isolated *ore* mutants with delayed leaf senescence by screening dark-incubated detached leaves. Among the mutants isolated 2 were allelic to *ein2*, further indicating the importance of ethylene's involvement in leaf senescence. *ORE9* has been cloned and shown to encode an F-box protein suggesting the involvement of the ubiquitin-mediated degradation of proteins in the regulation of leaf senescence (Woo et al., 2001). Using a similar dark screen approach, Yoshida et al. (2002a) isolated the *hys1* mutant that shows early leaf senescence phenotypes. *hys1* turned out to be allelic to the *cpr5* mutants, which were initially isolated in screens for altered pathogen-defence responses (Bowling et al., 1997) and for trichome mutants (Kirik et al., 2001).

As discussed above, the nature of ethylene's involvement in leaf senescence makes it an excellent means for the isolation of senescence mutants. We exploited the responses of *Arabidopsis* leaves to ethylene and found that ethylene promotes senescence within a specific age window. By utilising this knowledge, we have isolated mutants that are collectively named as *onset of leaf death* (*old*) mutants and describe here the characterisation of mutants with early leaf senescence phenotypes. The phenotypic, physiological, and molecular genetic characteristics of the mutants show that we have identified novel mutations. The importance and the positions of the *OLD* genes in the senescence-regulating network are discussed, and a genetic model that links the control of leaf senescence by *OLD* genes and the action of ethylene is proposed.

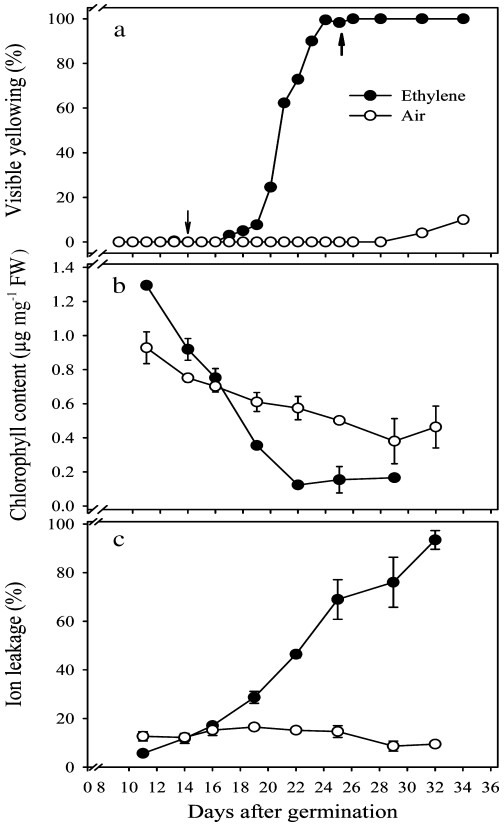

Figure 1. The effect of ethylene on visible yellowing, chlorophyll content and ion leakage of *Arabidopsis* cotyledons.
L*er*-0 plants were grown either continuously in air for the indicated days, or first in air until 3 d before the indicated days and then exposed to 10µll^{-1} ethylene. Cotyledon yellowing (**a**) was subsequently scored as the percentage of yellow cotyledons versus the total number of cotyledons and chlorophyll contents (**b**) and ion leakage (**c**) quantified. Cotyledons with over 10% yellow area of the blade were judged as yellow (corresponding to S1 described by Lohman et al., 1994). The visible yellowing was observed on at least 100 plants for each data point and the results for chlorophyll content and ion leakage were shown as mean±sd of three replicates. Arrows in (**a**) indicate the time points when the screen for early or late leaf senescence mutants was performed at the end of the 3-d ethylene exposure. Note that the initial drop of chlorophyll content in (**b**) coincided with the expanding cotyledons until 16 days.

Results

Ethylene treatment of mutagenised *Arabidopsis* plants allows for the isolation of leaf senescence mutants

As a first step towards establishing a mutant screen system, we tested the effect of a 3-d ethylene treatment on the progression of leaf senescence. Figure 1 shows the effect of ethylene treatment on the visible yellowing, chlorophyll content and ion leakage of *Arabidopsis* cotyledons. In air-grown plants visible yellowing appeared on cotyledons from 30 d onwards (Figure 1a), and all the plants had yellow cotyledons after 45 d (data not shown). Upon ethylene treatment, visible yellowing was not observed in plants younger than 17 d. Thereafter visible yellowing was induced in cotyledons and the cotyledons became yellow in all the plants older than 24 d. Thus exogenous ethylene does promote the cotyledon yellowing, but its effect depends on the age of cotyledons. Similar trends of visible yellowing were sequentially observed in the rosette leaves. For example, in ethylene-treated first rosette leaves yellowing started from 24 d and all the first rosette leaves became yellow after 28 d, while in the ethylene-treated second rosette leaves yellowing started at 25 d and they all became yellow after 29 d (data not shown). These results showed that ethylene induces visible yellowing of *Arabidopsis* cotyledons and leaves in an age-dependent manner.

Table 1. Genetic segregation analysis of *old* mutants

Male	Female	Generation	Wild-type	Mutant	χ^2	P
old1-1	L*er*-0	F_1	17	0		
		F_2	599	217	1.105	>0.29
old1-1	*old1-2*	F_1	0	29		
old1-1	*old1-3*	F_1	0	22		
old2	L*er*-0	F_1[a]	0	17		
		F_2	97	237	2.910	>0.08
old2	Col-0	F_1	54	0		
		F_2	168	190	1.469[b]	>0.22
old3[c]	L*er*-0	F_1	6	7		
		F_2	245	482/231[d]	0.447[e]	>0.79
old3[c]	Col-0	F_1	140	0		
		F_2	355	128/30[d]	0.136[f]	>0.93

[a]The *old2* heterozygotes had phenotypes similar to that of the parental *old2* homozygote. The phenotype scoring was carried out on 24-d-old plants treated with 10 µl l^{-1} ethylene for 3 d. The criteria were: wild-type plants with 0-3 yellow cotyledons and/or leaves, *old2* plants with 2 yellow cotyledons and at least 2 yellow leaves.

[b]A 7:9 ratio of wild-type:*old2* was tested.

[c]The pollen from *old3* heterozygotes was used for pollination since the *old3* homozygotes were lethal. Two types of F_1 plants were obtained in the back-crosses to Ler-0, whereas in the back-crosses to Col-0 all the F_1 progeny showed the wild-type phenotypes.

[d]The progeny of the *OLD3/old3* F_1 plants were used for segregation test. These numbers represent heterozygous/homozygous *old3*.

[e]A 1:2:1 ratio of Wild-type:intermediate:mutant was tested.

[f]A 11:4:1ratio of wild-type:intermediate:mutant was tested.

To further validate whether the effect of exogenous ethylene in inducing senescence was a function of leaf age, we followed changes in chlorophyll content and ion leakage. As shown in Figure 1b and 1c, a drop in the chlorophyll content and an increase in the ion leakage were associated with the progression of the visible yellowing, suggesting a well-defined correlation between the visible symptoms and the physiological changes. Interestingly, a vast increase in the ion leakage occurred after the chlorophyll content dropped to the minimal, showing that the occurrence of the cellular membrane damage lags behind the chlorophyll degradation. Similar results were reported by Woo et al. (2001). Taken together, these results showed that there is an intrinsic linkage among visible yellowing, chlorophyll degradation, and cellular membrane damage in ethylene-induced senescence and confirmed that the effect of ethylene on leaf senescence relies on leaf age (Grbic and Bleecker, 1995; Weaver et al. 1998).

The above experiments provided evidence for the following notions. (1) *Arabidopsis* cotyledons have to reach a defined developmental stage to perceive ethylene's action in inducing senescence. (2) An ethylene-treated mutagenised plant that shows yellow cotyledons before 17 d or no yellow symptoms after 24 d, can be considered a putative early or late leaf senescence mutant, respectively. These notions led to the establishment of a screen system for isolating leaf senescence mutants. Both early and late leaf senescence mutants were isolated as described in Experimental procedures and are names as *old* (*onset of leaf death*) mutants. In this paper, we focus on early leaf senescence mutants and report the

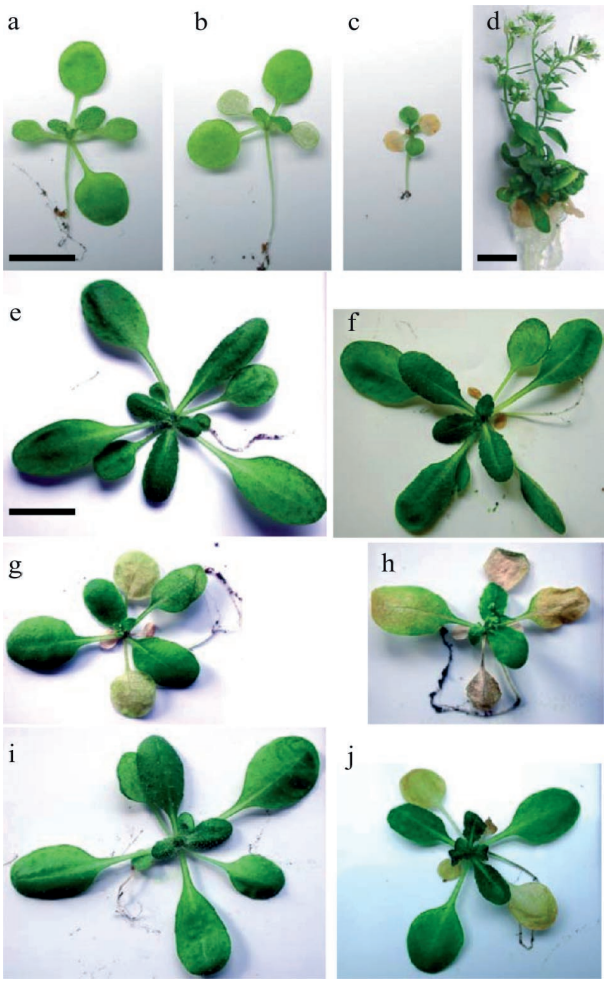

Figure 2. Phenotypes of old mutants in comparison with the wild-type.
(a-c) 16-d air-grown L*er*-0, *old1-1*, and *old3*.
(d) 50-d *old*3 grown in Murashige and Skoog medium plus 1% sucrose.
(e-f) 24-d L*er*-0 grown in air or with 3-d ethylene treatment.
(g-h) 24-d *old1-1* grown in air or with 3-d ethylene treatment.
(i-j) 24-d *old2* grown in air or with 3-d ethylene treatment.
The bar in a represents 0.5 cm and those in d and e represent 1 cm. Pictures a to c and e to j were taken at the same magnification.

characterisation of mutations in 3 genetic loci that are implicated in the regulation of leaf senescence.

Early leaf senescence mutants fall into 3 complementation groups

The early senescence mutants were sub-grouped into three classes, depending on changes in their phenotypes before and after ethylene treatment in comparison with the wild-type. Class I mutants show early senescence symptoms in air, and the symptoms are further enhanced by ethylene treatment. Class II mutants do not have early senescence phenotypes in air, but they exhibit accelerated senescence upon ethylene exposure. Class III mutants senesce earlier than wild-type in air, but ethylene treatment does not induce senescence in additional leaves. We selected representative mutants of each class from the L*er*-0 accession for further study. Back-cross and complementation tests placed them into three loci: *old1*, *old2* and *old3* (Table 1).

The *old1* locus is represented by 3 recessive alleles isolated from independent M_2 pools. We selected *old1-1* for future studies. The *old1-1* plants have a strong early leaf senescence phenotype. After ~14 d of growth, *old1-1* cotyledons became yellow, while wild-type cotyledons were still green (Figures 2a and 2b). By 24 d yellowing was observed in the first 2 rosette leaves as well, and ethylene treatment strongly promoted leaf senescence in *old1-1* plants (Figures 2g and 2h). The adult *old1-1* plants start bolting and flowering earlier (bolts arose in *old1-1* with 4-6 rosette leaves and in L*er*-0 with 8-9 rosette leaves) and had a reduced size and less progeny. The *old1-1* mutation was mapped to the bottom of chromosome 5, ~3 centiMorgans south of the single nucleotide polymorphism (SNP) marker SGCSNP84.

The *old2* plants are indistinguishable from wild-type plants in air (Figures 2e and 2i), but ethylene triggers accelerated senescence. After ethylene treatment at 21-24 d, *old2* plants showed yellow cotyledons and first 2 rosette leaves, whilst wild-type plants had yellow cotyledons (Figures 2f and 2j). Genetic analysis revealed that *old2* is an ethylene-required dominant trait and that there is a dominant suppressor of *old2* in Col-0 (Table 1). These characteristics make mapping a rather time-consuming process. The *old2* mutant belongs to Class II early leaf senescence mutants, and among 12 mutants isolated 6 showed similar phenotypes and complex genetic behaviour, including the presence of a dominant suppressor in Col-0. It seems likely that they represent the same locus, but this needs to be confirmed by further genetic analysis.

One single *old3* allele was identified. When grown in soil, the homozygous *old3* mutant is rosette-lethal and plants stop growing when the second pair of rosette leaves appears (Figure 2c). Interestingly, *old3* can be rescued by culture on medium with 1% sucrose, although the plants still senesce earlier (Figure 2d). Ethylene treatment enhanced the speed of senescence in cotyledons but did not induce yellowing in additional leaves. A heterozygous plant with intermediate *old3* phenotypes was back-crossed to the parental line L*er*-0, and the progeny tests showed that *old3* is a semi-dominant allele (Table 1). In all the F_1 plants of an *old3* heterozygote by Col-0 cross, the wild-type phenotype was observed indicating that a suppressor exists in Col-0. Subsequent observations of 39 independent F_2 populations showed that 20 of them contained the *old3* mutation and the segregation ratio manifested a duplication of a semi-dominant gene in Col-0. Plants homozygous for both the suppressor and the *old3* loci were used for mapping, and the two loci were placed at the lower arm of chromosome 3, ~4 centiMorgans south of simple sequence length polymorphic (SSLP) marker K11J14, and at the upper arm of chromosome 4, ~3.6 centiMorgans north of CAPS marker G4539a.

Thus, mutants with diverse early leaf senescence phenotypes were isolated, and genetic analysis indicated the presence of at least 3 genetic loci that are involved in the regulation of leaf senescence.

The senescence syndrome is advanced in *old* mutants

We set up and performed mutant screen based on the senescence phenotypes of cotyledons due to the advantage that the screen could be done in a large scale within limited space and a relatively short time. Initial analyses of the three mutants indicated that the earlier senescence also sequentially occurred in rosette leaves. Thus, we carried out further physiological and molecular characterisation of the senescence syndrome using cotyledons, specific rosette leaves and the whole rosette, respectively.

To examine the physiological changes during senescence, the chlorophyll content and the ion leakage were measured in whole rosette, cotyledons and different rosette leaves from 11-

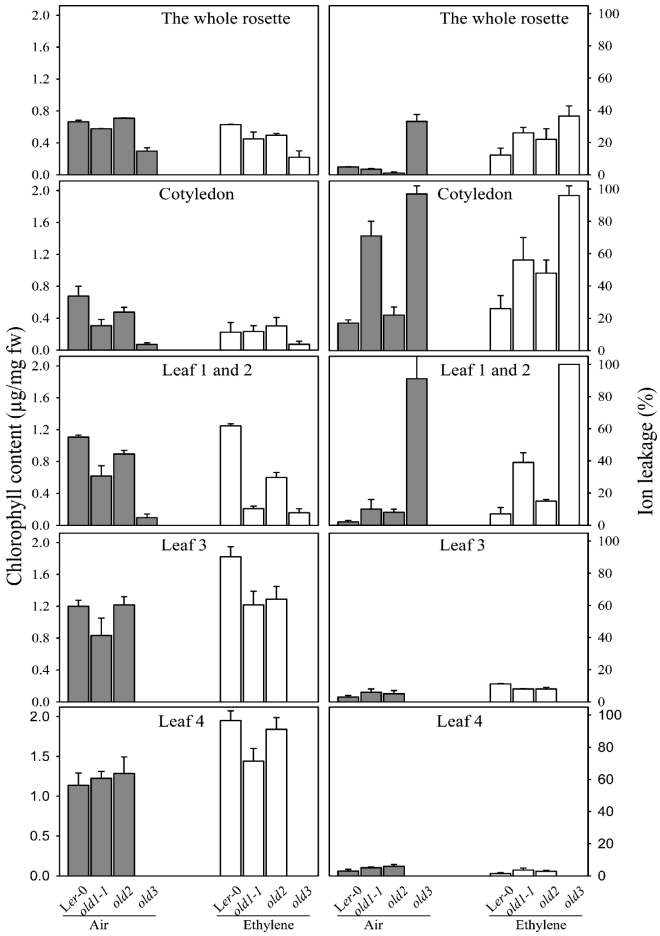

Figure 3. Comparison of chlorophyll content and ion leakage between the wild type and the three *old* mutants. L*er*-0 plants and *old* mutants were grown either in air for 24 d, or first in air until 21 d and then exposed to 10 µl l^{-1} ethylene for 3 d, and the whole rosette or leaves at specific positions were isolated and the chlorophyll content and the ion leakage quantified. Results are shown as mean±sd of at least three replicates. Note the chlorophyll content results between ethylene treatment and air were not comparable due to the inhibitory effect of ethylene on cell expansion.

d, 16-d, 24-d, 30-d and 40-d-old plants. Results for the 24-d samples are presented in Figure 3. In the whole rosette samples isolated from air-grown seedlings, a lower chlorophyll content was found in *old1* and *old3*, while a higher ion leakage was only observed in *old3*, but not in the *old1-1* samples. No differences were found between *old2* and the wild-type. Upon ethylene treatment, samples of the *old* plants showed lower chlorophyll content and higher ion leakage compared to the wild-type. These results were consistent with the visible yellowing of the mutants and the effect of ethylene in inducing visible yellowing (Figure 2), suggesting an overall acceleration of leaf senescence in the *old* mutants.

More detailed analyses using cotyledons, the rosette leaves 1/2, 3 and 4 also showed the advanced leaf senescence in the *old* mutants. For air-grown samples, the chlorophyll content and ion leakage of *old1-1* and *old3* were significantly different from those of *old2* and the wild-type, except the rosette leaf 4 from *old1-1*. Upon ethylene treatment, the chlorophyll content of cotyledons dropped to the minimal for all the plants and no differences were observed, but the ion leakage of the *old* mutants was still higher than that of wild-types. In ethylene-treated rosette leaves 1/2 large differences were observed between *old* mutants and

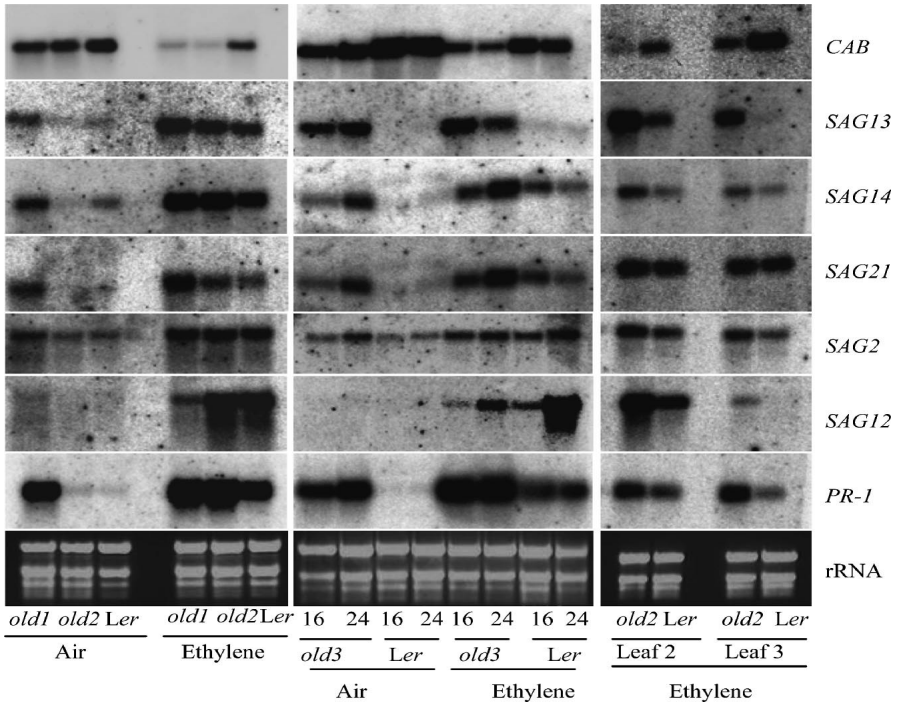

Figure 4. Northern hybridisation analysis showing the abundance of the mRNA of various genes in *old* mutants and wild-type plants.

The left column shows RNA samples from the whole rosette of 24-d *old1-1*, *old2* and the wild-type (L*er*-0) plants. The middle column shows RNA samples from the whole rosette of 16-d and 24-d *old3* and the wild-type plants. The right column shows RNA samples from the rosette leaf number 2 and the green leaf number 3 of the 24-d *old2* and wild-type plants with a 3-d ethylene treatment. For samples in the left and the middle columns 10 µg total RNA was loaded per lane, and for samples in the right column 5 µg RNA was loaded. The sources for each gene are shown in Table 2.

Table 2. Genes used fro hybridisation experiments

Gene names	Encoded protein	References
CAB	Chlorophyll a/b binding protein	Leutwiler et al., 1986
PR-1	Acidic pathogen-related protein	Uknes et al., 1992
SAG2	Cysteine protease	Hensel et al., 1993
SAG12	Cysteine protease	Lohman et al., 1994
SAG13	Short chain alcohol dehydrogenase	Lohman et al., 1994
SAG14	Blue copper-binding protein	Lohman et al., 1994; Weaver et al., 1998
SAG21	Late embryogenesis associated protein	Weaver et al., 1998

the wild-type for both parameters, but in rosette leaves 3 and 4 the *old* mutants differed from the wild-type in the chlorophyll content, not in the ion leakage, further showing the sequential occurrence of chlorophyll degradation and membrane damage. Together, lower chlorophyll contents and higher amounts of ion leakage were observed in air-grown *old1-1* and *old3* and in all ethylene-treated *old* samples, and the younger the leaf, the less the differences between

the *old* mutants and the wild-type, indicating that leaf senescence in the *old* mutants occurred earlier and was controlled by leaf age. These results also suggested that although the mutants were isolated on the basis of the altered senescence phenotypes of cotyledons, the senescence of the rosette leaves was similarly affected.

We subsequently performed a detailed molecular characterisation by Northern blot analysis of the mRNA abundance of the genes listed in Table 2. Changes in the mRNA levels of the tested genes fell into distinct classes (Figure 4). The first class is represented by *CAB* whose mRNA levels reduce during senescence. The levels of *CAB* mRNA were already lower in air-grown *old* plants and more strongly reduced upon ethylene treatment. The second class includes *SAG13*, *SAG14* and *SAG21*. In air, the mRNA levels of these genes in *old1* and *old3* were higher than those in *old2* and the wild-type. Upon ethylene treatment, more pronounced increases in the mRNA levels of these *SAG*s were observed in the *old* mutants in comparison with the wild-type. The third class is shown by *SAG2*, which showed a basal mRNA level that was higher in air-grown *old1* and *old3* plants, consistent with the early occurrence of senescence in these two *old* mutants. Upon ethylene treatment, the *SAG2* mRNA levels did not appear to be higher in *old* mutants, although the mutants showed stronger senescence. The mRNA level of *SAG2* is also increased by exogenous ethylene in green leaves besides being up-regulated during senescence (Hensel et al., 1993; Grbic and Bleecker, 1995), which might be the reason why in ethylene-treated samples little difference was detected between *old* mutants and the wild-type plants.

Changes in the *SAG12* mRNA levels distinctly differed from all the other tested *SAG*s and formed one class on its own. Like in the wild-type, air-grown *old2* plants showed no detectable level of *SAG12* mRNA as expected, but the *SAG12* mRNA levels were very low in air-grown *old1* and undetectable in *old3*, both having prominent senescence symptoms. Upon ethylene treatment, *old2* appeared to have a slightly higher level of *SAG12* mRNA than the wild-type. However, *old1* and *old3* showed lower levels of *SAG12* mRNA than the wild-type, which was again unexpected since much stronger senescence symptoms were evident in these two *old* plants.

Genes conferring resistance to pathogen attack are also highly expressed during senescence (Quirino et al., 1999; Morris et al., 2000). Thus we analysed the expression profile of *PR-1* in *old* mutants. Changes in the *PR-1* mRNA levels showed similar patterns to those of *SAG13*, *SAG14* and *SAG21*, high in air-grown *old1* and *old3* but similarly low in air-grown *old2* and the wild-type. Compared to the wild-type, the mRNA levels increased to much higher levels in ethylene-treated *old* mutants. Therefore, *PR-1* expression was enhanced in the *old* mutants.

The particular phenotype of *old2* mutants prompted us to perform more detailed gene expression analysis on ethylene-treated leaves. The 21-d *old2* and wild-type plants were exposed to ethylene for 3 d, and the *CAB* and *SAG* mRNA levels from the second and third rosette leaves analysed. In this experiment, over 95% of the second rosette leaves were still green in the wild-type after ethylene treatment, whereas over 90% showed signs of senescence in the *old2* plants. For the third rosette leaves only the green ones were used for both *old2* and the wild-type, although ~50% of the *old2* third rosette leaves were yellow. Compared to the wild-type controls, *old2* leaves had lower levels of *CAB* mRNA and higher mRNA levels of the tested *SAG*s. Crucially, even in the green third rosette leaves, the mRNA levels of the tested genes showed large differences between *old2* and the wild-type. In particular, the mRNA levels of *SAG12* and *SAG13*, which are believed to be good markers for natural leaf

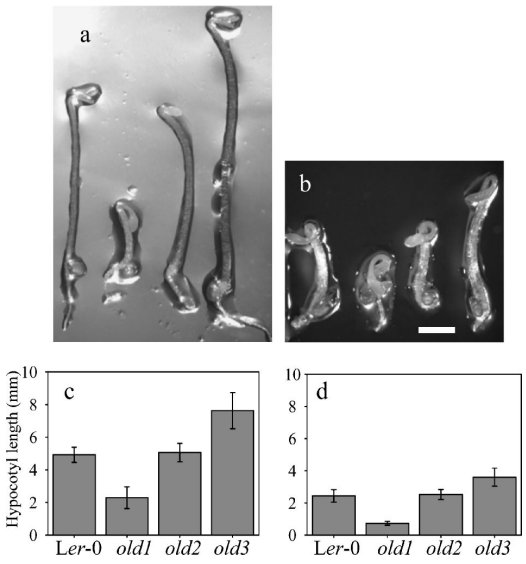

Figure 5. The triple response in the wild-type (L*er*-0) and *old* mutants.
(a) Wild-type and *old* seedlings in the absence of ACC.
(b) Wild-type and *old* seedlings in the presence of 10μM ACC.
(c) The hypocotyl length of wild-type and *old* seedlings in the absence of ACC.
(d) The hypocotyl length of wild-type and *old* seedlings in the presence of 10μM ACC. Seeds were surface sterilised with 25% bleach, plated on to Murashige and Skoog media with and without 10 μM ACC, and grown in darkness at 21°C for 5 d. The hypocotyl length was measured using at least 50 seedlings per treatment and shown as mean±sd.

senescence (Weaver et al., 1998), were prominent in *old2* but undetectable in the wild-type. This comparison more clearly demonstrated the advancement of leaf senescence in *old2*.

old mutants exhibit different ethylene responses

The progression of leaf senescence in the *old* mutants exhibited distinct patterns before and after ethylene treatment, thus the ethylene-signalling pathway was further studied in these *old* mutants by the triple response bioassay (Figure 5). The etiolated *old* mutants already displayed differences in hypocotyl length even in the absence of ACC (Figure 5a and 5c). *old1-1* seedlings showed a shorter hypocotyl. The hypocotyl of *old3* was longer than that of the wild-type, while there was no significant difference between *old2* and the wild-type. When 10 μM ACC was added, *old1-1* displayed an extremely exaggerated triple response, the whole seedling formed a ring-like shape (Figure 5b and 5d). At this ACC concentration, *old3* persistently showed a longer hypocotyl, whilst the hypocotyl of *old2* was not different from that of wild-type. These results suggest that *old1-1* may be hypersensitive to ethylene, *old3* responds to ethylene but sustains a longer hypocotyl, and the triple response is not altered in *old2*.

The *old* mutants may work in a common regulatory pathway

Since the three *old* mutants showed early senescence phenotypes and similar *SAG* expression profiles, they may work in a common regulatory pathway controlling leaf senescence. We performed double mutant analysis to determine the potential genetic interactions between the *OLD* genes. As shown in Table3, when *old1-1* was crossed to *old2*, the plants with *old1-1* phenotypes segregated in a 1:3 ratio; while in the F_2 plants of an *old1-1* by *old3* cross plants showing *old1-1* and *old3* phenotypes segregated in a 3:4 ratio, which suggests that *OLD1* might work upstream of *OLD3* and downstream of *OLD2*. Like *old3* the *old1-1/old3* double mutant is also rosette-lethal. The results from testing relationship between *old2* and *old3*

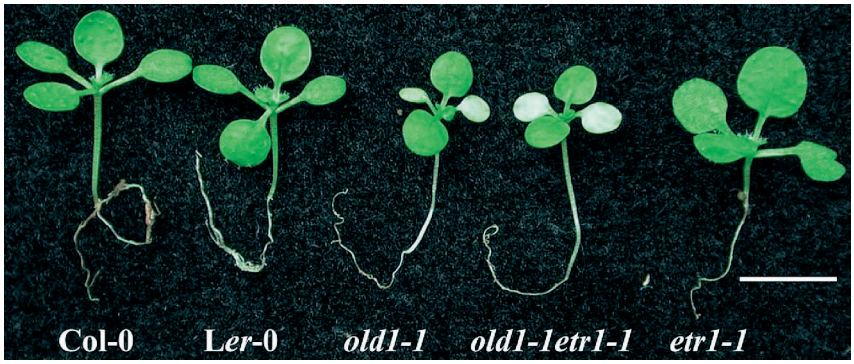

Figure 6. Phenotypes of *old1-1*, *etr1-1* and *old1-1etr1-1* mutants.
Seedlings were grown in conditions described in Experimental procedures for 17 d and photographed. Bar represents 1 cm.

Table 3. Segregation analysis of F_2 progeny of crosses among three *old* mutants

Crosses	WT	*old1-1*	*old2*	He-*old3*	H-*old3*	χ^2	P
old1-1xold2	51	87	163	-	-	2.59[a]	>0.27
old1-1xold3	75	102[b]	-	203	124	5.69[c]	>0.12
old2xold3	13	-	48	148	80	3.04[d]	>0.38

Phenotype scoring was detailed in Experimental procedures. WT: wild type; He: heterozygous; H: homozygous
a: A 3:4:9 ratio of WT:*old1-1*:*old2* was tested.
b: *old1-1old1-1/OLD3old3* and *old1-1old1-1/OLD3OLD3* showed *old1-1* phenotypes.
c: A 3:3:6:4 ratio of WT:*old1-1*:He-*old3*:H-*old3* was tested.
d: A 1:3:8:4 ratio of WT:*old2*:He-*old3*:H-*old3* was tested.

were also consistent with putting *OLD3* downstream of *OLD2*. Overall, results of the epistatic analysis correlated well with the phenotypes of the mutants and the results from *SAG* expression analysis, implying that the *OLD* genes may control a common regulatory pathway for leaf senescence.

Owing to the particular phenotypes of *old1-1*, we considered that *OLD1* may act at the linking point between ethylene- and age-regulated leaf senescence and constructed the double mutant of *old1-1* and *etr1-1*. As shown in Figure 6, at 17 d the air grown *old1-1etr1-1* showed senescing cotyledons as in the *old1-1*, suggesting that the earlier onset of leaf senescence in *old1-1* is independent of ethylene perception. On the other hand, when treated with ethylene, the double mutant failed to show a more exaggerated senescence syndrome as in the *old1-1* indicating that the progression of leaf senescence in *old1-1* does require a functional ethylene pathway. Taken together, these results suggest that *OLD1* mediates the actions of ethylene and age-related factors in the regulation of leaf senescence.

Discussion

The senescence window

In an attempt to identify genetic loci that define the onset of leaf senescence, the interaction between ethylene and leaf age was studied in *Arabidopsis*. The effect of ethylene in inducing

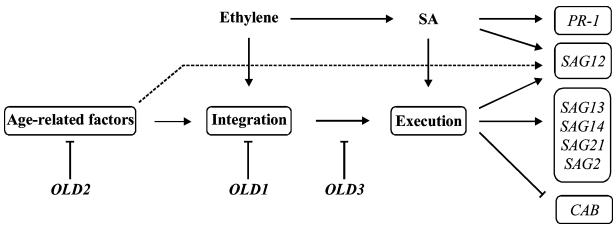

Figure 7. A genetic pathway showing the proposed positions of the *OLD* genes in the regulation of leaf senescence.

The model is constructed by analysing the phenotypes, genetics and *SAG* expression of the old mutants. The emphasis is on the control of *OLD* genes and their interaction with ethylene. An arrow indicates a stimulatory effect, while a T-bar represents an inhibitory effect. See text for details.

senescence varied largely, depending on the time when it was applied. Our results further confirmed the notion that ethylene can only induce senescence after a leaf reaches a certain developmental stage (Grbic and Bleecker, 1995; Weaver et al., 1998).

In extreme situations such as in the *ctr* plants in which ethylene signalling is constantly switched on, or in wild-type plants that are grown in the continuous presence of exogenous ethylene, early leaf senescence is not induced (data not shown, Kieber et al., 1993). In opposite situations such as in the *ein* mutants in which the ethylene signalling is permanently blocked, or in the plants with anti-sense constructs that reduce ethylene biosynthesis, normal leaf senescence eventually occurs (Bleecker et al., 1988; Grbic and Bleecker, 1995; John et al., 1995; Chao et al., 1997; Oh et al., 1997; Alonso et al., 1999). These observations exhibit that ethylene has no effect in stimulating senescence before or after certain developmental stages. In conclusion, a leaf has a defined age window to perceive the effect of ethylene on senescence, which is termed here as the senescence window.

Ethylene is involved in almost every façade of plant's development and responses to various stresses (reviewed by Johnson and Ecker, 1998). Ethylene response windows have also been described for other developmental events. Raz and Ecker (1999) showed that ethylene treatment does not affect the curvature of the apical hook in etiolated seedlings when given before or after certain growth stages. The effects of ethylene in releasing dormancy and promoting germination depend on the concentrations of exogenous ABA (Beaudoin et al., 2000). In tomato's susceptible response to pathogen infection, ethylene is required for the development of disease symptoms but is not essential for restricting pathogen infection and the primary lesion formation (Lund et al., 1998). In tomato, ethylene treatment can induce epinasty in the young and middle-aged leaves, but not in older ones (Abeles et al., 1992). Tomato fruit ripening was induced by exogenously-applied ethylene in mature green fruits, but not in immature fruits (Yang, 1987). These observations suggest that many such windows exist in plants to perceive ethylene action. When these windows open and how wide the windows are, may depend on the physiological context, developmental stage and the genetic make-up.

Many genes are involved in the biosynthesis, signalling and action of ethylene and are differentially regulated. In *Arabidopsis*, 5 genes encoding ACC synthase are differentially

expressed in plant organs, during development and in response to various stress conditions (Liang et al., 1992). Throughout plant development, the expression of the *ETR* ethylene perception gene family is differentially regulated in tomato (Lashbrook et al., 1998). In both *Arabidopsis* and tomato a large gene family of *EIN3* and *EIL* was identified (Chao et al., 1997; Tieman et al., 2001). Downstream are ethylene-response-factor1 and ethylene-responsive element binding factors whose differential regulation is also observed (Solano et al., 1998; Fujimoto et al., 2000). Such a sophisticated biosynthesis and signalling pathway is consistent with the existence of the diverse ethylene response windows.

The understanding of the relationship between the age-related factor and ethylene allowed us to formulate a model for the regulation of leaf senescence as shown in Figure 7. In this model, age-related factors are proposed to control the senescence window of a leaf and ethylene has to be recognised and integrated with the ageing signals. Perception of the signals leads to the activation of *SAG*s to accomplish senescence. This model provides clear implications: mutations causing perturbations in the control of age-related factors, in the integration of age and ethylene signals, or in the execution process of senescence may give predicted phenotypes. By utilising this knowledge and defining the senescence window, a screening system was established and mutants with altered leaf senescence phenotypes were isolated.

Functions of *OLD* genes

Bearing the model in mind, we tried to select those mutants in our collection with various senescence phenotypes that might locate at different positions in the regulating pathway. The phenotypes of the *old1*, *old2* and *old3* suggested that they potentially represented genes acting at the proposed positions (Figure 7). The physiological and molecular analyses were consistent with the suggested positions of the *OLD* genes, which were further confirmed by genetic interaction analysis.

The unique phenotype of *old2* suggests that in *old2* mutants the components of the regulatory pathway from ethylene signalling to the execution of senescence are not affected. Indeed, dark-grown *old2* seedlings showed a normal triple response. The *SAG* expression profile of ethylene-treated *old2* plants was not different from that of the wild-type, but advanced. Epistatic analysis also suggested that *OLD2* might work upstream of *OLD1* and *OLD3*. The simplest explanation could be that the *old2* mutation abolishes suppression on age-related factors and shifts the ethylene response window to an earlier stage. Thus, we infer that *OLD2* acts as a repressor of age-related factors upstream of ethylene action.

Air-grown *old1-1* plants display a strongly advanced senescence syndrome, which was further accelerated by ethylene treatment, suggesting that *old1* mutation generates alterations in two sets of pathways: age-regulated leaf senescence and ethylene signalling. This was more convincingly shown by the phenotypes of the *old1-1* and *old1-1etr1-1* double mutant. When ethylene perception was blocked in *old1-1*, the earlier onset of leaf senescence still occurred, but was not exaggerated by ethylene treatment. Thus, a plausible explanation for *old1-1* phenotypes would be that *OLD1* acts as repressor of the integration process.

It is important to notice that the *old1* mutants were different from *ctr* and *eto* that show a constitutive ethylene response (Guzmàn and Ecker, 1990; Kieber et al., 1993). *OLD1* is likely to define a novel link integrating ethylene actions into leaf senescence. Ethylene is shown to interact with many other signalling molecules such as with ABA in seed germination (Beaudoin et al., 2000), with jasmonic acid in conferring defence response (Penninckx et al., 1996), with

glucose in regulating seedling growth (Zhou et al., 1998), or with light in controlling hypocotyl elongation (Smalle et al., 1997). Isolation and analysis of *OLD1* may provide important insights on the interaction between senescence and ethylene signalling pathways.

In *old3*, leaf senescence occurred very rapidly, and ethylene treatment did not induce additional new leaves to senesce, suggesting that onset of leaf senescence in *old3* is independent of ethylene. We also constructed the *old3etr1-1* double mutants and observed that the lethal *old3* phenotype was not affected when the *etr1-1* mutation was introduced (data not shown). Therefore, *OLD3* may act downstream of ethylene at a late step of the senescence-regulating network with pleitrophic functions, which is consistent with the double mutant analysis that placed *OLD3* downstream of *OLD1*. One bottleneck of isolating early leaf senescence mutants is that mutations in homeostatic or housekeeping genes could also give an early senescence or lethal phenotype, which may not be distinguishable from the mutations in genes that specifically regulate leaf senescence. For instance, the premature senescence or lethal phenotype is generated from a mutation in a copper transport gene (Woeste and Kieber, 2000). It is hard to distinguish whether the lethal phenotype is the cause of the rapidly accelerated senescence or the consequence. Nevertheless, the lethality of *old3* can be prevented by culture in sucrose but the senescence is still accelerated in *old3*. This may argue against the first possibility. Together, we infer that *OLD3* may be a pleitrophic regulatory gene acting at a late step of the senescence-regulating networks.

In *Arabidopsis*, *SAG12* expression is highly associated with age-regulated senescence and is not induced by several stress conditions and believed to be a reliable marker for natural leaf senescence (Lohman et al., 1994; Weaver et al., 1998; Noh and Amasino, 1999). In *old1-1* and *old3*, *SAG12* consistently showed lower levels of mRNA upon ethylene treatment in comparison with the wild-type. These results raise two points. First, it is clear that high *SAG12* expression is not essential for the progression of leaf senescence in *old1-1* and *old3*. Second, these two *OLD* genes are likely to control a common pathway that is required but is not sufficient for the full expression of *SAG12* in wild-type plants. Disruption of *OLD2* alone did not induce *SAG12* expression. However, *old2* mutants showed higher levels of *SAG12* expression than *old1-1*, *old3* and the wild-type when treated with ethylene. This suggests that in *old2* mutants all the pathways required for full expression of *SAG12* are intact and active. In other words, *OLD2* controls an additional pathway that is involved in regulation of *SAG12* expression besides the *OLD1-OLD3* pathway. Therefore, analysis of *SAG12* expression in *old* mutants discloses at least two parallel regulatory pathways.

Previously, several factors required for the expression of *SAG12* have been identified. Noh and Amasino (1999) proposed the existence of a transcription factor that can bind to the senescence-specific promoter region of *SAG12* and initiate *SAG12* transcription in senescing leaves. Morris et al. (2000) showed that SA is also required for the expression of *SAG12*. The inducible *PR-1* expression demonstrated that the SA-mediated pathway is present in the *old* mutants. Thus *OLD* genes may be not involved in the SA-signalling pathway *per se*. Also, *OLD* gene products are unlikely to be the transcription factors only acting on *SAG12*, since a broad spectrum of SAG expression was altered. Nevertheless, it is clear that *OLD* genes are required for *SAG12* expression. Thus, we propose at least three different pathways to regulate *SAG12* expression in the model.

In summary, we have presented a model in an attempt to explain the control of senescence by age-related factors, the integration of ethylene action, and the regulation of SAG expression during the execution process. In contrast to the wealth of knowledge on the ethylene signalling

pathway, our current information on the molecular mechanisms of the regulation of leaf senescence and on the involvement of ethylene in senescence is scarce. Our model, although rudimentary, matches well the current knowledge available. The phenotypic, genetic and *SAG* expression analysis of the mutants provided information concerning at which steps the genes act and allowed for the construction of the model. Further refinements, through the isolation of mutants in additional steps, the analysis on double mutants from the *OLD* and the ethylene signalling pathways, and cloning and characterisation of the *OLD* genes, should yield more elaborate models for the regulation of leaf senescence.

Experimental procedures

Plant lines and growth conditions

Arabidopsis Landsberg *erecta* (L*er*-0) was the parental line for mutagenesis. Plants were grown in a growth chamber at 21°C and 70% relative humidity under ~60 $\mu molm^{-2}sec^{-1}$ fluorescent and incandescent light. Day length was set at 16h. An organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) was used for plant growth after sterilisation and drying. Plants for ethylene exposure were treated in a flow-through chamber at 20°C and ~50% relative humidity under continuous illumination. For experiments involving plants grown under sterile conditions, seeds were surface-sterilised with 25% commercial bleach and plated on Murashige and Skoog medium solidified with 0.8% agar.

Seed mutagenesis and isolation of mutants

Approximately, 20,000 seeds were treated with 0.3% ethyl methanesulfonate (EMS) for 15 h as previously described by Dijkwel et al. (1997). M_1 (mutagenised generation 1) seeds were sown on 500 pots of 20 cm in diameter, and seeds produced by 20-30 plants per pot were individually harvested and formed independent pools.

For each M_2 pool ~300 seeds were sown on a 20 by 30 cm tray. M_2 plants were grown for 11 d in air, treated with 10$\mu l\ l^{-1}$ ethylene (AGA, The Netherlands) for 3 d, and plants with yellowing cotyledons selected. The remaining plants were allowed to grow further in air for 7 d and treated with ethylene between 21-24 d, and plants with no signs of yellowing were sought. In total, ~150,000 plants were screened, and ~700 putative mutants were isolated. M_3 plants were further re-screened twice to eliminate false positives. Eventually, 62 mutants from 54 pools were obtained. The mutant lines were crossed twice to L*er*-0 before molecular and physiological characterisation.

Genetic analysis

For mapping, the *old* mutants were crossed to Col-0, and at least 30 F_2 plants homozygous for the *old* mutations were selected for DNA isolation using the SHORTY quick preparation (http://www.biotech.wisc.edu/*Arabidopsis*/). Linkage of the *old* mutations to cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994) and single nucleotide polymorphsim (SNP) (Drenkard et al., 2000) markers was analysed.

To test the genetic interactions among the three *OLD* genes, *old1-1* pollen was used to pollinate *old2* and pollen from heterozygous *old3* plants was used to pollinate *old1-1* or *old2*. To check the segregation of the *old1-1* by *old3* cross, F_2 seedlings were grown in the aforementioned climate room for 14 d and scored for *old1-1* and the lethal *old3* phenotypes.

At 21 d the heterozygous *old3* seedlings were easily distinguished from the wild type. For segregation analysis of the *old1-1* by *old2* cross, F_2 seedlings were first grown in air, scored for *old1-1* phenotypes and subsequently transferred to ethylene chamber at 21 d. After a 3-d ethylene treatment, *old2* plants were with yellow cotyledons and 2-4 rosette leaves, which were distinguished from wild-type plants that showed yellow cotyledons plus 1 rosette leaf at the most. Similarly, the homozygous and heterozygous *old3* plants and the *old2* plants were scored in the F_2 progeny of the *old2* by *old3* cross.

To generate double mutants of *old1-1* and *etr1-1*, the *old1-1* pollen was used. The F_2 population was grown in soil and seedlings showing *old1-1* phenotypes were selected to check the presence of the *etr1-1* mutation using a PCR marker (Hua and Meyerowitz, 1998). Since *old1-1* was isolated from L*er*-0 and *etr1-1* was from Col-0, we also used the SNPs to genotype *old1-1etr1-1*. The F3 progeny was used for experiments.

Measurements of chlorophyll content and ion leakage

Individual leaves or whole rosettes were taken from at least 6 seedlings. For measuring the chlorophyll content, samples were incubated overnight in 80% (v/v) aceton at 4°C in darkness, and the chlorophyll content quantified spectrophotometrically using the method of Inskeep and Bloom (1985). For measuring ion leakage, leaf samples were immersed into deionised carbonate-free water, shaken in a 25°C water bath for 30 min, and the conductivity was measured using a Wissenschaftlich Technische Werkstatten conductivity meter (model KLE1/T, , Weilheim, Germany). Then samples were boiled for 10 min and conductivity measured again. The percentage of the first measurement over the second measurement was used as the membrane damage indicator.

RNA extraction and Northern blot analysis

Plant material was frozen in liquid nitrogen and total RNA was isolated and quantified spectrophotometrically. For RNA blot analysis, 5 or 10 µg of RNA samples were separated by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde and then capillary-blotted onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). Ethidium bromide was included in the sample-loading buffer at a concentration of 4 µg/ml to confirm equal sample loading and blotting. Probes described in Table 2 were ^{32}P labelled by random-priming, and hybridisation was done at 65°C overnight in the Church and Gilbert (1984) buffer, and the membranes were washed once in 2x SSC for 5 min and twice for 15 min each in 0.1x SSC and 0.1% SDS at 65°C. Probe hybridisation was visualised with a phosphorimager using the OptiQuant software (Canberra Packard, Zellik, Belgium). Stripping of the membranes was done by boiling in 0.5% SDS solution for 3 min, and each membrane was used for probing maximally three times.

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